Glycobiology vol. 7 no. 4 pp. 453–461, 1997

Synthesis of a new nanomolar saccharide inhibitor of lymphocyte adhesion: different polylactosamine backbones present multiple sialyl Lewis x determinants to L-selectin in high-affinity mode

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Lymphocyte infiltration is a hallmark of acute rejections in solid organ transplants, such as cardiac allograft. We have previously shown that lymphocyte extravasation to cardiac grafts undergoing rejection is largely due to interactions between lymphocyte L-selectin and its sialyl Lewis x (sLex) decorated ligands. Our previous work demonstrated further that an enzymatically synthetized tetravalent sLex glycan of a branched polylactosamine backbone is a highly efficient inhibitor of L-selectin-dependent lymphocyte adhesion to graft endothelium. To improve the availability of multivalent sLex glycans for anti-inflammatory indications, we now report enzymatic synthesis of another tetravalent sLex glycan that can be potentially produced on a large scale, and show that even the new saccharide is a nanomolar inhibitor of L-selectin-dependent lymphocyte adhesion. The novel antagonist is sLexβ1–3′(sLexβ1–6′)LacNAcβ1–3′(sLexβ1–6′)LacNAcβ1–3′(sLexβ1–6′)LacNAc (8) (where LacNAc is the disaccharide Galβ1–4GlcNAc and sLex is the tetrasaccharide Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc). Its five-step synthesis was started from the octameric polylactosamine LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)LacNAc (3), which in turn is accessible in one step from the hexasaccharide LacNAcβ1–3′LacNAcβ1–3′LacNAc. Importantly, the hexasaccharide primer has been synthesized chemically (Alais and Veyrieres, Tetrahedron Lett., 24, 5223, 1983). Hence, our data outline a route to glycan 8, consisting of a combination of chemical and enzymatic methods of oligosaccharide synthesis. In addition, our data show that polylactosamine backbones are able to present multiple sialyl Lewis x determinants to L-selectin in high-affinity mode, without a requirement for uniqueness in the backbone structure.

Key words: synthesis/polylactosaminoglycans/multivalent/inhibitors/selectin

Introduction

Leukocyte tethering to and rolling on endothelial cells, mediated by the interaction of members of the selectin family and their oligosaccharide-bearing counterreceptors, initiates the extravasation (Hogg and Berlin, 1995; Ley and Tedder, 1995; Springer, 1995). L-Selectin is expressed on leukocytes and recognizes endothelial mucins GlyCAM-1, CD-34 and MAdCAM-1, which carry oligosaccharides of sialyl Lewis x (sLex) or sulfated sLex type (Hemmerich and Rosen, 1994; Hemmerich *et al.*, 1994b, 1995; Lasky, 1995; McEver *et al.*, 1995). The primary role of L-selectin is to guide lymphocyte extravasation into peripheral lymphoid tissues, and we have previously shown that peripheral lymph node high endothelium expresses selectively sLex epitopes (Paavonen and Renkonen, 1992). *De novo* expression of sLex oligosaccharides on vascular endothelium leads to enhanced lymphocyte adhesion to endothelium also at sites of inflammation in a L-selectin–dependent manner (Turunen *et al.*, 1994, 1995). Exogenous monovalent sLex glycans have been shown to inhibit selectin-dependent inflammations both *in vitro* and *in vivo* in animal models (Mulligan *et al.*, 1993; Buerke *et al.*, 1994; Rao *et al.*, 1994; DeFrees *et al.*, 1995; Han *et al.*, 1995).

Recently, we described enzyme-assisted synthesis of a tetravalent 22-meric sLex glycan (saccharide 2 in Figure 1), derived from a *branched* polylactosamine backbone (Seppo *et al.*, 1996). It proved to be a more potent inhibitor of L-selectin–mediated lymphocyte adhesion to endothelium than the monovalent sLex tetrasaccharide (Seppo *et al.*, 1996; Turunen *et al.*, 1995). The control glycan (saccharide 1 in Figure 1), lacking the fucose residues, had no effect on the lymphocyte binding. This suggests that the L-selectin–dependent lymphocyte extravasation can be prevented specifically and efficiently by the presence of exogenous multivalent sLex polylactosamines.

Appropriate *in vivo* experiments would provide an obvious way to test this conclusion. However, the synthesis of the glycan 2 appears too complicated to allow construction of samples large enough for such experiments at present time. Therefore, we have now worked towards chemo-enzymatic methods of the synthesis of polylactosamine glycans carrying multiple sLex units.

Here, we show that a novel tetravalent sLex glycan (saccharide 8 of Figure 2), derived from a *linear* polylactosamine backbone, is also a powerful inhibitor of L-selectin–mediated cell adhesion. The novel L-selectin antagonist was derived from a linear polylactosamine primer, itself accessible by chemical synthesis (Alais and Veyrieres, 1983). In the present experiments, glycan 8 was still synthesized using purely enzymatic methods, but its production by a combination of chemical and enzymatic reactions appears promising, because in this approach several particularly difficult steps involving the β1,3-GlcNAc transferase reaction can be avoided.

Results

Enzymatic synthesis of glycan 8

An outline of the synthesis route employed is shown in Figure 2. The yields of the individual glycosyltransferase reactions in our first experiment are indicated in Table I.

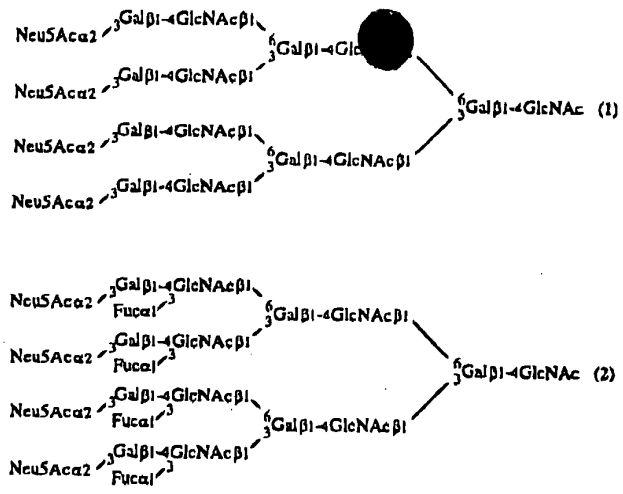


Fig. 1. Structures of glycans 1 and 2. The latter represents a tetravalent sialyl Lewis x glycan of a *branched* backbone, and was shown to be a high affinity antagonist of L-selectin (Turunen *et al.*, 1995; Seppo *et al.*, 1996). In the present experiments, the antagonist properties of glycan 2 were shown to be considerable even at concentrations smaller than the previously reported ones. The fucose-free glycan 1 revealed no antagonist activity.

The octasaccharide **3** (150 nmol) was first elongated in a β1,3-GlcNAc transferase reaction. The resulting nonasaccharide **4** was isolated as a mixture with some unreacted glycan **3** by Superdex 75 HR chromatography, and subjected as such to a reaction catalyzed by β1,6-GlcNAc transferase from hog gastric mucosa. The reaction mixture, containing glycan **5** and other oligosaccharides, gave two Superdex HR 75 peaks, containing 612 nmol and 215 nmol of bound GlcNAc, respectively. The composition of these peaks was analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Stahl *et al.*, 1991; Harvey, 1993). The principle Superdex peak, eluting at 60.94 min, had two major components: One, $(M+Na)^+$ m/z 1949.5 (65 mol %), represented $GlcNAc_7Gal_3$ (**5**; calc. m/z 1949.8) while the other, $(M+Na)^+$ m/z 1746.7 (30 mol %), represented $GlcNAc_6Gal_3$ (calc. m/z 1746.6). In the minor Superdex peak, at 62.94 min, the most abundant ion in the molecular ion region of the mass spe[illegible] had $(M+Na)^+$ m/z 1380.5 (monoisotopic), indicatin[illegible]t this fraction contained 70 mol % $GlcNAc_5Gal_2$ (calc. monoisotopic m/z 1380.5). Low abundance signals of monoisotopic m/z 1177.4, 1542.5, and 1746.0 could be assigned to the presence of $GlcNAc_4Gal_2$, $GlcNAc_5Gal_3$ and $GlcNAc_6Gal_3$ species, respectively (calculated monoisotopic values m/z 1177.4, 1542.6, and 1745.6). The complex mixture that was obtained during the synthesis of glycan **5** was generated by β-galactosidase and β-N-acetylglucosaminidase present in the crude β1,6-GlcNAc transferase extract (Helin *et al.*, 1993).

Exhaustive β1,4-galactosyl transferase reaction converted glycan **5** into glycan **6**, the branched array of seven LacNAc units. Superdex 75 HR chromatography of the reaction product revealed a well-shaped oligosaccharide peak. MALDI-TOF mass spectrum of the peak material showed signals at m/z 2598.1, assigned as $(M+Na)^+$ of $GlcNAc_7Gal_7$ (**6**; calc. m/z 2598.4) (65 mol %) and m/z 2233.0, assigned as $(M+Na)^+$ of $GlcNAc_6Gal_6$ (calc. m/z 2233.0) (35 mol %).

Exhaustive α2,3-sialyltransferase reaction converted glycan **6** to glycan **7**. The product was isolated by chromatography on Superdex 75 HR in a nearly pure form. Ensuing anion ex-

Table I. Yields in the stepwise conversion of glycan 3 to glycan 8[a]

Glycan	Yield	
	nmol	%
3	150	100
4	ND	58[b]
5	62[c]	41
6	53[c]	35
7	49[d]	33
8	31[e]	21

[a]Calculated values, including also the samples used for analytical purposes. Actually, only 24 nmol of glycan **8** were obtained.
[b]Data from another experiment.
[c]Estimated from the apparent GlcNAc-content of the appropriate peak in Superdex HR 75 chromatography and from the peak composition deduced from MALDI-TOF mass spectrometry.
[d]Estimated from the apparent content of $(Neu5Ac)_4 + (GlcNAc)_7$ in the appropriate peak from MonoQ-chromatography.
[e]Estimated from the apparent content of $(Neu5Ac)_4 + (GlcNAc)_7$ in the appropriate peak from HPAE-PAD-chromatography.

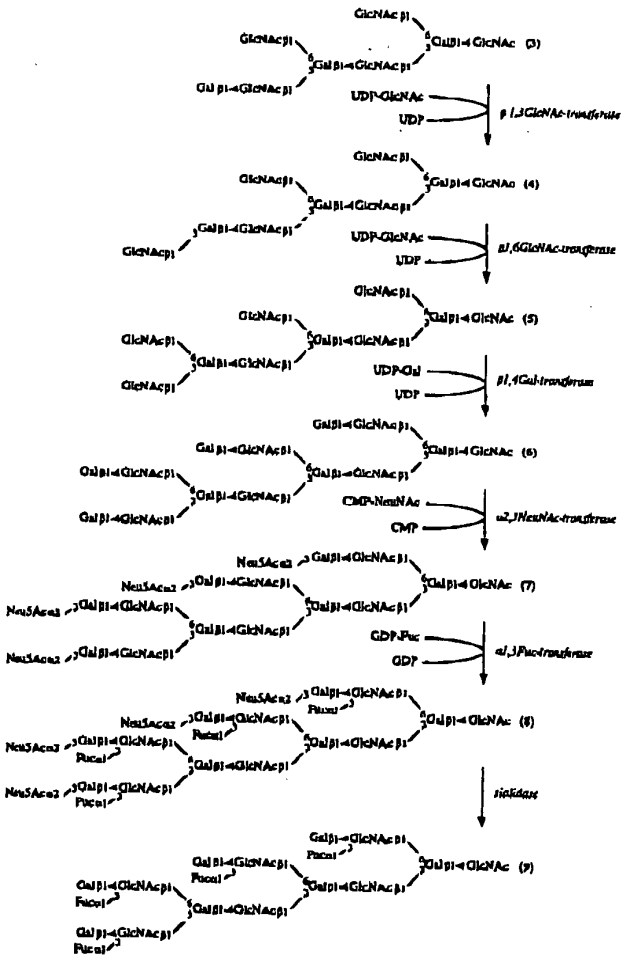


Fig. 2. Outline of the present synthesis route from glycan 3 to the tetravalent sialyl Lewis x saccharide 8 of a *linear* backbone, and further to the Lewis x saccharide 9.

change chromatography on a Mon[redacted]column gave the tetrasialoglycan **7** in a pure form (Figu[redacted]). Glycan **7** chromatographed in these experiments, and also in HPAE-PAD chromatography on a Dionex CarboPac PA-1 column, like the isomeric tetrasialylated glycan **1** (Seppo *et al.*, 1996), and was distinctly slower than a trisialylated marker saccharide in the MonoQ experiment. ^{1}H-NMR spectrum of glycan **7** at 500 MHz confirmed its structure. In particular, reporter signals of four equivalents of α2,3-bonded N-acetyl-neuraminic acid and four equivalents of the sialylated galactose residues 6, 12, 17, and 18 were present in the spectrum (Figure 4A, Table II).

Exhaustive α1,3-fucosyltransferase reaction converted glycan **7** to the tetravalent sLex glycan **8**. Preliminary purification of glycan **8** was effected by chromatography on Superdex 75 HR. Subsequent HPAE-PAD chromatography gave glycan **8** as a well-shaped peak at 8 ml (Figure 3B). Desalting of the peak on the Superdex 75 HR column gave 24 nmol of pure glycan

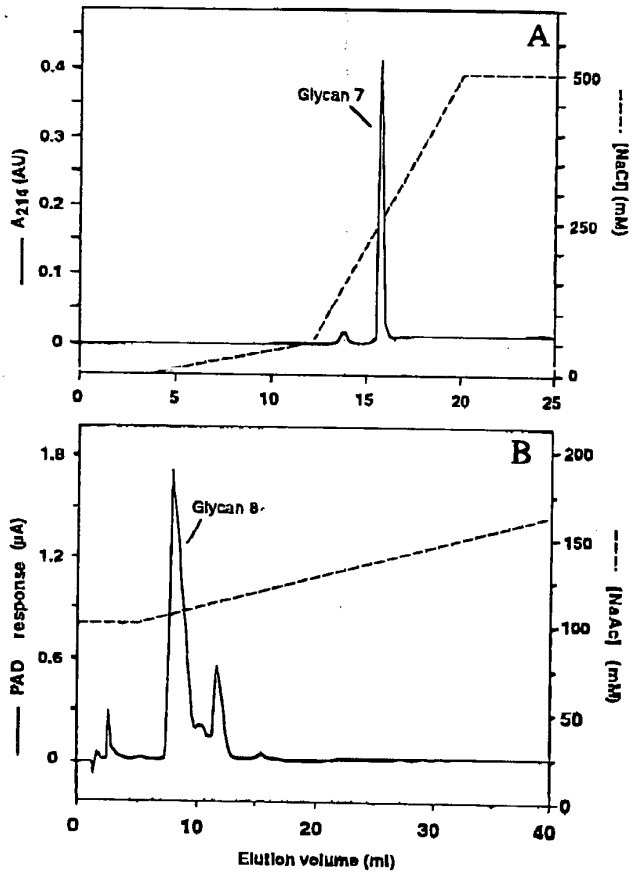


Fig. 3. (A) Anion exchange (MonoQ) chromatography of partially purified tetrasialylated oligosaccharide derived from glycan **6**. The major peak, eluting at 16 ml represented glycan **7**, the smaller peak at 14 ml probably represented $Neu5Ac_3Gal_6GlcNAc_6$. Glycan **7** chromatographed here like glycan **1** (Seppo *et al.*, 1996), an isomeric tetrasialo-polylactosamine, and was well separated from a more rapidly emerging trisialylated oligosaccharide marker. (B) HPAE-PAD chromatography of crude glycan **8**, isolated from the synthesis mixture by gel filtration. The major peak eluting at 8 ml represents glycan **8**, while the peak at 12 ml is believed to contain the reducing-end-ManNAc analog. The latter was probably formed by base-catalyzed epimerization at C2 of the reducing-end-GlcNAc of glycan **8** and/or the precursors. In parallel experiments glycan **8** chromatographed like the isomeric glycan **2**.

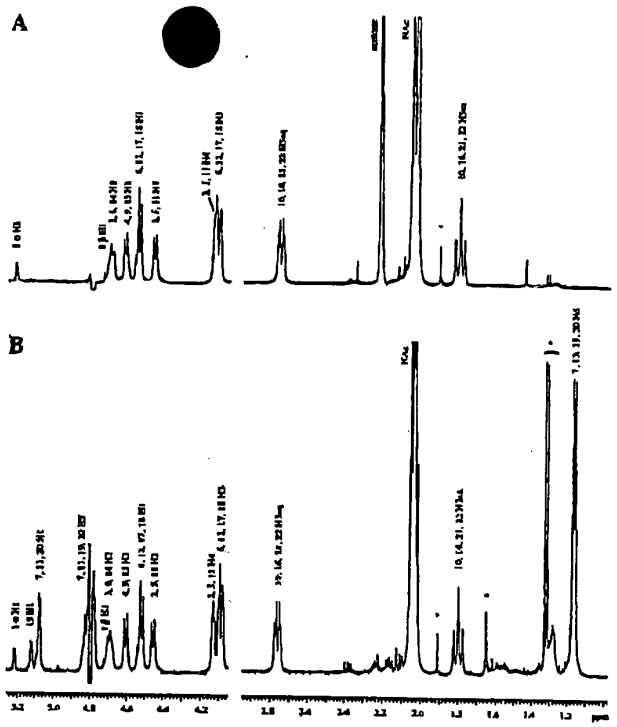


Fig. 4. (A) Expansion of ^{1}H-NMR spectrum of glycan **7**. (B) Expansion of ^{1}H-NMR spectrum of glycan **8**. The resonances marked by an asterisk arise from unknown impurities.

8. The presence of four fucosyl residues reduced the affinity of glycan **8** to the CarboPac PA-1 column dramatically, compared to glycan **7**. This is characteristic of fucosylated saccharides (Hardy and Townsend, 1989). Parallel experiments revealed that the isomeric glycans **8** and **2** (see Figures 1 and 2 for the structures) co-chromatographed on CarboPac PA-1. In contrast, the trifucosyl analog of glycan **2** (Seppo *et al.*, 1996) emerged from the column much later, at 16 ml. Remarkably, the α1,3-fucosyltransferase reaction of glycan **7** appeared to proceed more easily to completion than the analogous reaction with the isomeric glycan **1** (Seppo *et al.*, 1996).

Another complete synthesis experiment was also performed by starting from 400 nmol of glycan **3**. No difficulty was experienced in the scaling up, and at the end 150 nmol of pure glycan **8** were actually obtained.

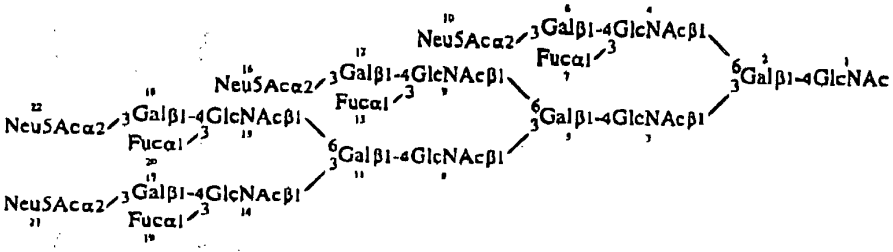
Structural characterization of glycan 8

^{1}H-NMR spectrum of glycan **8** (Figure 4B, Table II) confirms the structure. Besides the reducing end GlcNAc, H-1 signals of three β1,3-linked GlcNAc residues at 4.684–4.696 ppm are visible and accompanied by H-1 signals of three β1,6-bonded GlcNAc units at 4.603 ppm. The six GlcNAc residues are bonded to three galactoses, which reveal H-4 resonances at 4.133 ppm, the characteristic region for a H-4 resonance of a galactose that is disubstituted by GlcNAc units at positions 3 and 6 (Koenderman *et al.*, 1987). The galactose H-1 region reveals signals of the three branching galactoses at 4.452 ppm, those of sialylated galactoses of the three β1,6-bonded sLex determinants at 4.517 ppm and that of the β1,3-bonded sLex at 4.533 ppm. The H-3 signals of the sialylated galactoses are characteristically (Kamerling and Vliegenthart, 1992) at 4.089

Table II. ^{1}H-NMR chemical shifts of struct[illegible]porter groups of glycans 7 and 8 at 23°C

Residue	n:o[a]	Proton	Glycan	
			7	8
GlcNAc	1 (α)	H-1	5.214	5.214
GlcNAc	1 (β)	H-1	4.725	4.713
Gal	2,5,11	H-1	4.458	4.452
		H-4	4.143	4.133
GlcNAc[b]	3	H-1	4.691	4.684/4.687
GlcNAc[b]	4	H-1	4.606/4.612	4.603
GlcNAc	8,14	H-1	4.691	4.596
GlcNAc	9,15	H-1	4.620	4.603
Gal	6,12,18	H-1	4.544	4.517
Gal	17	H-1	4.558	4.533
	6,12,17,18	H-3	4.119	4.089
Fuc	7,13,20	H-1	—	5.076
		H-5	—	4.820
		H-6	—	1.166
Fuc	19	H-1	—	5.119
		H-5	—	4.820
		H-6	—	1.166
Neu5Ac	10,16,21,22	$H\text{-}3_{ax}$	1.803	1.798
		$H\text{-}3_{eq}$	2.756	2.762

[a]Numbering of the residues is as follows:



[b]The two chemical shift values given arise from signals representing the α- and β-pyranosic forms of the glycan.

ppm. The equatorial and axial H-3 resonances of Neu5Ac at 2.762 and 1.798 ppm, respectively, confirm the presence of four equivalents of α2,3-bonded Neu5Ac (Kamerling and Vliegenthart, 1992). The signals of the methyl protons at 2.04 ppm corresponded to the presence of 11 N-acetyl groups. The H-1 of the fucose residue in the β1,3-bonded sLex determinant resonated at 5.119 ppm, while those of the three β1,6-bonded sLex units resonated at 5.076 ppm. The H-5 and H-6 signals of the fucoses resonated characteristically (Vliegenthart *et al.*, 1983; de Vries *et al.*, 1993) at 4.820 and 1.166 ppm, respectively. The integrals of the H-1 and H-6 protons indicated the presence of four fucoses.

Because underivatized sialoglycoconjugates are fragmented during MALDI-TOF mass spectrometry (Juhasz and Costello, 1992), glycan **8** was not subjected directly to MALDI-TOF analysis. However, the presence of four fucose residues in glycan **8** was confirmed by MALDI-TOF mass spectrometry after prior removal of the sialic acid residues. For this, a sample of glycan **8** was treated with *Arthrobacterium ureafaciens* sialidase. The desalted reaction mixture was subjected to MonoQ chromatography, which yielded the neutral asialo-glycan **9**. In the MALDI-TOF mass spectrum of glycan **9**, a major $(M+Na)^+$-peak, representing 80 mol % of the polylactosamine signals, was observed at m/z 3182.8 (calculated for $Fuc_4Gal_7GlcNAc_7$, 3182.9). Two minor components, evident in the spectrum, behaved as $Fuc_3Gal_7GlcNAc_7$ (12%) and $Fuc_3Gal_6GlcNAc_6$ (8%). The minor signals may represent degradation products generated during desialylation or mass spectrometry, because repeated HPAE-PAD chromatographic runs of intact **8** on CarboPac PA-1 failed to reveal significant amounts of material eluting at 16 ml, around the expected position of $Neu5Ac_4Fuc_3Gal_7GlcNAc_7$.

Glycan **9** resisted jack bean β-galactosidase treatment, which is characteristic of terminal Galβ1–4(Fucα1–3)GlcNAc sequences (Kobata, 1979). The unchanged MALDI-TOF mass spectrum had the $Fuc_4Gal_7GlcNAc_7$ $(M+Na)^+$ signal (calc. m/z 3182.9) as the major component, measured at m/z 3182.8 before and m/z 3183.2 after the treatment. Hence, all fucose residues of glycan **8** were bonded to the distally located, sialylated N-acetyllactosamine units. The data confirm and extend our previous findings, showing that α1,3/4-fucosyltransferase from human milk does not react with LacNAc residues that carry branches at the 6′-position (Maaheimo *et al.*, 1995; Niemelä *et al.*, 1995; Seppo *et al.*, 1996).

Glycans 2 and 8 as inhibitors of L-selectin–dependent lymphocyte adhesion to endothelium

Finally, we compared in parallel experiments the capacity of the tetravalent sLex glycans **2** and **8**, and of the nonfucosylated analogs **1** and **7**, to inhibit L-selectin–dependent lymphocyte adhesion to cardiac endothelium during acute rejection. The lymphocytes were preincubated for 30 min with varying concentrations of the oligosaccharides and used thereafter in the Stamper–Woodruff binding assay in the incubation media

(Stamper and Woodruff, 1976). The [redacted] sLex bearing glycans 2 and 8 were effective in inhibiting [redacted]mphocyte adhesion to activated cardiac epithelium, the IC_{50}-values values being around 1 nM for both sLex glycans (Figure 5). The crucial control saccharides 1 and 7, having the same charge, the same overall structure and approximately the same size, but being devoid of fucose, did not alter the lymphocyte binding from background levels. This data indicates that both the branched and linear tetravalent sLex glycans 2 and 8 are extremely efficient in inhibiting the L-selectin–dependent lymphocyte adhesion to endothelium and thereby should reduce the rejection associated inflammation. The synthesis of glycans 2 and 8 is possible by the fully enzymatic approaches, described by Seppo *et al.* (1996) and in the present experiments, respectively. Glycan 8, however, can be obtained also by a combined chemo-enzymatic approach, starting from the chemically synthesized hexasaccharide primer LacNAcβ1–3′LacNAcβ1–3′LacNAc (Alais and Veyrieres, 1983) as outlined by the present experiments and those of A. Leppänen *et al.* (unpublished observations); the latter providing a link between hexasaccharide primer LacNAcβ1–3′LacNAcβ1–3′LacNAc and glycan 3. We plan to continue studies of this kind with glycan 8, in order to reach a synthesis scale that is large enough to allow *in vivo* experiments with this L-selectin antagonist.

Discussion

Inflammation in rejecting solid organ transplants is characterized by heavy infiltration of lymphocytes into the tissue (Renkonen *et al.*, 1983). The lymphocytes must extravasate from blood circulation to reach the sites of inflammation. This process is initiated by a selectin–carbohydrate interaction, which results in lymphocyte rolling on the vascular endothelium, followed by firm adhesion and penetration through the vascular wall (Hogg and Berlin, 1995; Lasky, 1995; Ley and Tedder, 1995; McEver *et al.*, 1995; Nelson *et al.*, 1995; Springer, 1995; Whelan, 1996). L-Selectin is the only member of the selectin family that is expressed on the leukocyte surfaces. Its ligands

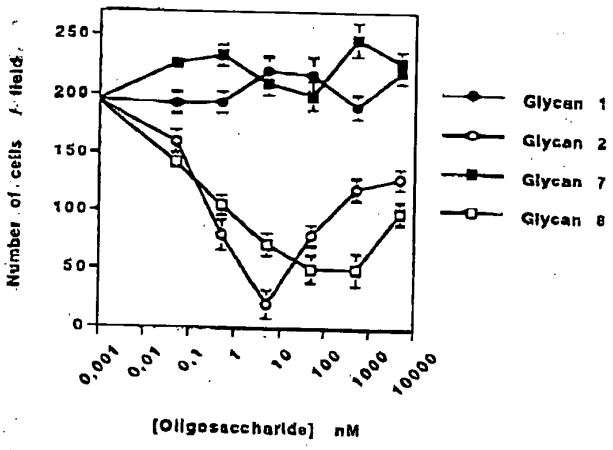


Fig. 5. L-Selectin–dependent binding of lymphocytes on endothelium of rejecting cardiac transplants of rats in the presence of synthetic oligosaccharides. The mean ± SEM of one representative experiment out of three is presented. The tetravalent sialyl Lewis glycans 8 and 2 inhibited the lymphocyte adhesion strongly, revealing IC_{50}-values values around 1 nM. The nonfucosylated analogs 7 and 1 revealed no inhibitory properties.

on endothelium [redacted]GlyCAM-1, CD34 and MAdCAM-1, which all are m[redacted]type glycoproteins decorated with O-linked sLex or sulfated sLex glycans (Rosen and Bertozzi, 1994; Bertozzi, 1995; Crottet *et al.*, 1996).

There is both *in vitro* and *in vivo* data indicating that the sLex-containing glycans can modify the leukocyte–endothelial interactions. In endothelial cell cultures sLex could inhibit the E-selectin–dependent adhesion of tumor cells to endothelium or P-selectin–dependent aggregation of platelets to target cells (Moore *et al.*, 1994; DeFrees *et al.*, 1995; McEver *et al.*, 1995). Reperfusion injuries, occurring when the blood supply to a given tissue is reconnected after a short interruption, lead to massive tissue necrosis. The necrosis is mainly caused by granulocytes, which extravasate to these tissues in a selectin-dependent manner. *In vivo* treatments by monovalent sLex glycan in several, but not all, animal models have shown the beneficial effects of this approach (Mulligan *et al.*, 1993; Buerke *et al.*, 1994; Han *et al.*, 1995).

We have initiated a program where we synthesize complex sLex-decorated polylactosaminoglycans, pursuing for increased affinity to L-selectin (Maaheimo *et al.*, 1995; Turunen *et al.*, 1995; Seppo *et al.*, 1996). The aim is to use these glycans as inhibitors of L-selectin–dependent cell adhesion and extravasation, leading to downregulation of inflammation. The present report describes enzymatic synthesis and characterization of the tetravalent sialyl Lewis x glycan 8 (for the structure, see Figure 2), which carries the sLex residues on a linear backbone of three LacNAc residues. The structural characterization of glycan 8 was performed by ^{1}H NMR-spectroscopy at 500 MHz, by chromatographic and degradative experiments, and by MALDI-TOF mass spectrometry, performed with the desialylated glycan 9. The biological effects of glycan 8 were monitored by using the well defined model of L-selectin–dependent lymphocyte adhesion to the activated endothelium of rejecting cardiac graft (Tamatani *et al.*, 1993; Turunen *et al.*, 1994, 1995; Seppo *et al.*, 1996).

The biological properties of the linear-backbone glycan 8 resembled those of the isomeric branched-backbone glycan 2 (for the structure, see Figure 1), which was synthesized previously in our laboratory (Seppo *et al.*, 1996). Both glycan 8 and glycan 2 were very potent L-selectin antagonists in the present Stamper–Woodruff adhesion experiments: glycan 8 revealed strong inhibitory effect down to 0.5 nM, while glycan 2 was, overall, even a somewhat stronger L-selectin antagonist (see Figure 5). It is remarkable that *both* glycan 8 and glycan 2 showed high-affinity binding to L-selectin. Obviously, neither unique backbone arrays nor rigidly defined positioning of the sLex determinants are required for high-affinity recognition by L-selectin; only the presence of several sLex determinants on linear or branched polylactosamine backbones appears to be important. The linear backbones have the advantage of having been synthesized chemically (see below). The role of the relatively rigid polylactosamine backbones in selectin antagonist activity may not be trivial, because flexible aliphatic backbones carrying multiple sLex units are not particularly effective antagonists (DeFrees *et al.*, 1993). The activities of glycans 8 and 2 were completely dependent on the presence of the intact sLex sequences in the binding determinants; the presence of the α1,3-bonded fucose residues was required for recognition. In addition to sLex-determinants, even sLex-related epitopes, bonded in multiple copies to polylactosamine backbones generate high affinity antagonists of L-selectin as shown by ongoing experiments in our laboratories.

The low nanomolar range of multivalent sLex glycans 2 and 8, reported here is several orders [redacted] the inhibitory range of monovalent sLex measured in the same assay (Turunen *et al.*, 1995). Other conventional high affinity inhibitors of L-selectin include mucins of endothelial and other origins (Imai *et al.*, 1991; Lasky *et al.*, 1992; Baumhueter *et al.*, 1993; Berg *et al.*, 1993; Imai and Rosen, 1993; Hemmerich and Rosen, 1994; Hemmerich *et al.*, 1994a, 1995; Crottet *et al.*, 1996). Interestingly, the O-linked oligosaccharides released from these mucins by alkaline borhydride did not show any detectable binding to L-selectin in affinity chromatography experiments (Crottet *et al.*, 1996). Our data show, however, that oligosaccharides of proper structure (i.e., those containing polylactosamine backbones decorated by multiple sLex groups) can be recognized by L-selectin with high affinity. Interestingly, the inhibitory activity of glycans 2 and 8 was highest at a distinct concentration, being partially abolished at lower as well as higher concentrations. At the high ligand concentrations, artefactual cross-reactivity and cell aggregation may have occurred.

The very high biological activity of glycans 8 and 2, compared to the monovalent sLex, is probably based on their multivalency. This situation is in many ways analogous to the binding of multivalent Neu5Acα2–6Galβ1–4GlcNAc ligands to CD22β, a sialic acid–specific lectin of B cells (Powell and Varki, 1994). The multivalent sLex glycans may crosslink two or several L-selectin molecules, known to be clustered on the tips of lymphocyte microvilli (Hasslen *et al.*, 1995; von Andrian *et al.*, 1995). The segmental flexibility of L-selectin would be helpful in the presentation of the carbohydrate recognizing domains (Rosen and Bertozzi, 1994), allowing crosslink-formation despite the vicinity of the individual sLex determinants in a given ligand molecule. The proximal ends of two sLex determinants of 2, for example, are at most only 2 nm apart, even in the maximally extended conformation of the polylactosamine backbone (Renouf and Hounsell, 1993). However, it has been argued (Crottet *et al.*, 1996) that selectin aggregation may not be important, because high-affinity binding to cell surfaces has been observed with soluble monomeric P-selectin (Ushiyama *et al.*, 1993) and E-selectin (Hensley *et al.*, 1994). Another possibility is that the multivalent sLex glycans acquire their high affinity by binding to two distinct sites within a L-selectin monomer. Recent data of Malhotra *et al.* (1996) suggest that the interaction of L-selectin and its endothelial ligands may require occupancy of both the sLex-recognizing site (CRS), which is probably monovalent, and a distinct adjacent binding site recognizing acidic determinants (ARS). This arrangement would be similar to the clustered patch, involving tyrosine sulfate residues immediately adjacent to sialylated oligosaccharides, generating P-selectin recognition in PSGL-1 (Sako *et al.*, 1995; Wilkins *et al.*, 1995). Hence, the possibility exists that the tetravalent sLex glycans 8 and 2 bind to monomeric L-selectin in two ways, a specific joint between one sLex-determinant and the CRS, and a less specific binding between the sialic acid of another sLex-residue in the ligand and the ARS of L-selectin. This notion would require that even partially fucosylated derivatives of the tetrasialoglycan 7, for instance, are particularly good adhesion inhibitors. Regardless of the binding mode, the saccharide antagonists of L-selectin, exemplified by glycans 8 and 2, are interesting as potential anti-inflammatory drugs, because they are presumably much less antigenic than the mucin or neoglycoprotein ligands of selectins (Welply *et al.*, 1994).

One may ask whether the remarkably low IC_{50}-values of glycans 2 and 8 observed in the present experiments, simply reflect some particular characteristics inherent in the binding assay of Stamper and Woodruff (1976), with which L-selectin was originally discovered (Siegelman *et al.*, 1989). At the moment, this notion appears unlikely. In fact, in a direct comparison the Stamper–Woodruff assay, involving the binding of lymphocytes to endothelium on frozen sections, gave higher IC_{50}-values for adhesion-inhibiting soluble ligands of L-selectin than a conventional ELISA competition assay (O'Connell *et al.*, 1996). We have used the demanding Stamper–Woodruff assay because of its specificity for L-selectin. The Ca^{2+}-dependent adhesion of lymphocytes to high endothelial cells of peripheral lymph nodes, under the conditions of this assay, is inhibited by a recombinant L-selectin–IgG chimera and also by a functional anti-rat-L-selectin MAb (Tamatani *et al.*, 1993). Likewise, in our own laboratory, 60–70 % of the site-specific lymphocyte binding to peritubular capillary endothelium of rejecting renal graft was inhibited by anti-sLex- and anti-L-selectin antibodies and also by the monovalent sLex tetrasaccharide (Turunen *et al.*, 1994). Also in rejecting cardiac grafts used in the present experiments, lymphocyte binding to the endothelium under these conditions is L-selectin mediated (Turunen *et al.*, 1995). Another important reason for the use of the Stamper–Woodruff assay in experimenting with our multivalent inhibitors is that in ELISA-type assays it is very difficult to mimic correctly the relevant patchwise presentation of L-selectin at the tips of microvilli on lymphocyte surfaces.

In addition to L-selectin–mediated processes, glycans 8 and 2 are likely to inhibit adhesion phenomena involving other selectins. For example, the data of Nelson *et al.* (Nelson *et al.*, 1992) suggest that in man, E-selectin–dependent adhesion may be even more effectively inhibited than L-selectin–mediated processes by sLex-saccharides. It remains to be seen whether the high efficiency of glycan 8 as L-selectin antagonist can be found also in *in vivo* experiments, which we plan to perform in transplantation models, where lymphocytes dominate in the inflammatory infiltrate.

In the present experiments, the tetravalent sLex glycan 3 was synthesized by using enzymatic steps only, and even the primer octasaccharide 3 (see Figure 2 for the structure) had been constructed enzymatically. However, glycan 3 can be obtained also in a chemo-enzymatic process, where the linear hexasaccharide LacNAcβ1–3′LacNAcβ1–3′LacNAc is constructed chemically using the block synthesis (Alais and Veyrieres, 1983,1987), and then transformed into glycan 3 using an enzymatic reaction catalyzed by a specific β1,6-GlcNAc transferase (A. Leppänen *et al.*, unpublished observations). For this reaction, the hexasaccharide 3 is incubated with UDP-GlcNAc and a β1,6-GlcNAc transferase (GlcNAc to Gal) acting at the centrally located galactoses of the acceptor chain. Hence, our data outline a chemo-enzymatic total synthesis of glycan 8, which profits the scaling-up potential of organic chemistry, and avoids two particularly difficult β1,3-GlcNAc transferase reactions inherent in the all-enzymatic approach.

Taken together, our data imply that (1) glycan 8 is a highly potent inhibitor of L-selectin–dependent lymphocyte adhesion to the activated endothelium of rejecting cardiac transplants of rats, and that (2) a chemo-enzymatic synthesis of glycan 8, which can be potentially performed on a large scale, is feasible. It remains to be seen whether it is possible to synthesize 8 in amounts that are large enough for the study of the anti-inflammatory potential of this glycan *in vivo*.

Materials and methods

Synthesis of the octasaccharide glycan 3

Glycan 3 (see Figure 2) was synthesized enzymatically as described elsewhere (A. Leppänen *et al.*, unpublished observations). In short, the hexasaccharide LacNAcβ1–3'LacNAcβ1–3'LacNAc was decorated by the two β1,6-bonded GlcNAc branches by incubating it with UDP-GlcNAc and the centrally acting β1,6-GlcNAc transferase (GlcNAc to Gal), present in rat serum (Gu *et al.*, 1992). The resulting glycan 3 was purified by chromatography and extensively characterized by degradative experiments as well as ^{1}H-NMR and MALDI-TOF mass spectrometry.

^{1}H-NMR-spectroscopy

Prior to NMR experiments the saccharides were twice lyophilized from ^2H_2O and then dissolved in 600 μl ^2H_2O (99.996%, Cambridge Isotope Laboratories, Woburn, MA). The NMR experiments were performed on a Varian Unity 500 spectrometer at 23°C. In recording the proton spectra, a modification of WEFT sequence (Hård *et al.*, 1992) was used. The ^{1}H chemical shifts were referenced to acetone, 2.225 ppm.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MALDI-TOF mass spectrometry was performed in the positive ion reflector mode with irradiation from a nitrogen laser (337 nm) and 2,5-dihydroxybenzoic acid as the matrix with the Finnigan Vision 2000 time-of-flight instrument (Thermo BioAnalysis, Ltd., Hemel Hempstead, UK), operated at 5 kV accelerating voltage and with 4 kV postacceleration at the detector. External calibration was used. Mass assignments are reported as average mass values, unless noted otherwise.

Transferase reactions

The reactions with hog gastric β1,6-GlcNAc transferase (Piller *et al.*, 1984), bovine milk β1,4-galactosyltransferase (Brew *et al.*, 1968), human serum β1,3-GlcNAc transferase (Yates and Watkins, 1983), human placenta α2,3-sialyltransferase (Nemansky and van den Eijnden, 1993), and human milk α1,3/4-fucosyltransferase (Eppenberger-Castori *et al.*, 1989; Natunen *et al.*, 1994) were performed essentially as described previously (Maaheimo *et al.*, 1995).

Chromatographic methods

Gel permeation chromatography on Superdex 75 HR (Pharmacia Sweden) was performed on two consecutive columns (10 × 300 mm) run at 0.5 ml/min with water (neutral saccharides) or 0.05 M NH_4HCO_3 (sialic acid–containing saccharides). The effluent was monitored at 214 nm, and the oligosaccharides were quantified against external GlcNAc and Neu5Ac.

For anion exchange chromatography, a MonoQ (5/5) column (Pharmacia) was eluted at a rate of 1 ml/min, first isocratically with water for 4 min, then with a linear gradient of 0 to 0.05 M NaCl over 8 min, and finally with a linear gradient of 0.05 to 0.5 M NaCl over 8 min. A trisialylated oligosaccharide, Neu5Acα2–6Galβ1–4GlcNAcβ1–2(Neu5Acα2–3Galβ1–4GlcNAcβ1–4)Manα1–3(Neu5Acα2–6Galβ1–4GlcNAcβ1–2Manα1–6)Manβ1–4GlcNAc, from Dr. G. Strecker (University of Lille, France), and the tetrasialylated polylactosamine glycan 1 (Figure 1) (Seppo *et al.*, 1996) were used as markers.

High-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed on a (4 × 250 mm) Dionex CarboPac PA-1 column at a flow rate of 1 ml/min., first isocratically with 100 mM sodium acetate in 100 mM NaOH for 5 min and then with a linear gradient of 100–200 mM sodium acetate in 100 mM NaOH over 55 min. The fractions collected were neutralized with 0.4 M aqueous acetic acid, and desalted by using gel permeation chromatography on Superdex HR 75.

Exoglycosidase digestions

For cleavage with *A.ureafaciens* sialidase (Boehringer, Mannheim, Germany), saccharide samples were incubated overnight with 80 mU of the enzyme in 40 μl of 100 mM sodium acetate, pH 5.0. Incubation with jack bean β-galactosidase was performed as described previously (Renkonen *et al.*, 1989). In parallel β-galactosidase reactions, the disaccharide (^{3}H)Galβ1–4GlcNAc was completely degraded, releasing (^{3}H)Gal.

Rats, transplantations and Stamper–Woodruff lymphocyte adhesion assay

Inbred WF (RT1^u) and DA (RT1^a) rat strains were carried in our own colony and regularly tested for intrastrain acceptance of cardiac and renal transplants as well as for the absence of intrastrain mixed lymphocyte culture. Rats of 10–12 weeks of age were used for the transplantations and a modified microvascular technique was used. Allogeneic (DA to WF) transplants were removed on day 3 after the transplantation. Small pieces of the removed hearts were mounted in Tissue Tek medium (Lab-Tek Productions Naperville, IL) and snap-frozen in liquid nitrogen. Frozen sections of 8 μm were prepared.

Single cell suspensions of mesentrical lymph node lymphocytes were prepared by mechanical disaggregation in RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 25 mM Hepes, pH 7.4, and 0.5% fetal calf serum, and the cells were passed through a 50 μm pore size mesh. The lymphocytes were of >99% purity, and the population consisted of 80–90% CD3-positive T cells, 50–60% CD4-positive T cells, 25–35 % CD8-positive T-cells, and 10–20 % CD19-positive B cells as analyzed by flow cytometry.

For the binding assay (Stamper and Woodruff, 1976; Turunen *et al.*, 1995), 3×10^6 cells in 100 μl of the medium were plated on top of the tissue sections, using a wax pen circle to keep the fluid in place. The slides were then rotated horizontally on a shaker at 60 r.p.m. for 30 min at +4°C. After incubation, the medium was gently removed by absorbent paper, and the slides were fixed in 1.5% cold glutaraldehyde overnight, and stained with thionine for 30 min. Excess thionine was washed away with phosphate-buffered saline (PBS), and the slides were mounted with PBS-glycerol (1:1). The number of bound lymphocytes were determined from 10 high-power fields/section. Each experiment involved incubation of lymphocytes with six individual sections of the rejecting heart and three independent experiments were carried out. To analyze the effect of oligosaccharides in the lymphocyte–endothelium binding assay, the lymphocytes were incubated with the saccharides for 30 min at 4°C prior to adding them to the sections.

Acknowledgments

This study was supported by grants from the Academy of Finland, the Rector of University of Helsinki, the Medical Faculty of University of Helsinki and the Technology Development Center, Finland.

Abbreviations

ARS, binding site of L-selectin that recognizes acidic determinants; CRS, sLex-recognizing site of L-selectin; Fuc, L-fucose; Gal, D-galactose; GlcNAc, N-acetyl-D-glucosamine; HPAEC, high pH anion exchange chromatography; MALDI, matrix-assisted laser desorption/ionization; Neu5Ac, N-acetylneuraminic acid; PAD, pulsed amperometric detection; sLea, sialyl Lewis a; sLex, sialyl Lewis x; TOF, time of flight.

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Received on June 25, 1996; revised on September 17 and December 5, 1996; accepted on December 14, 1996